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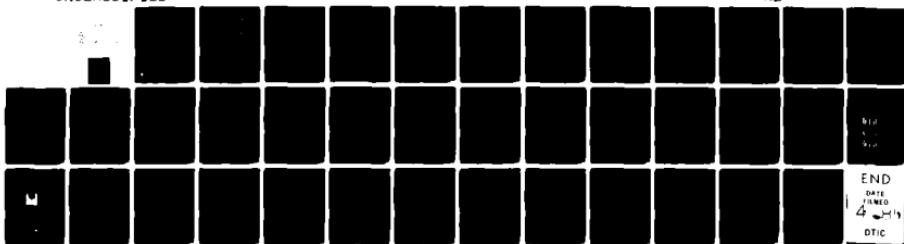
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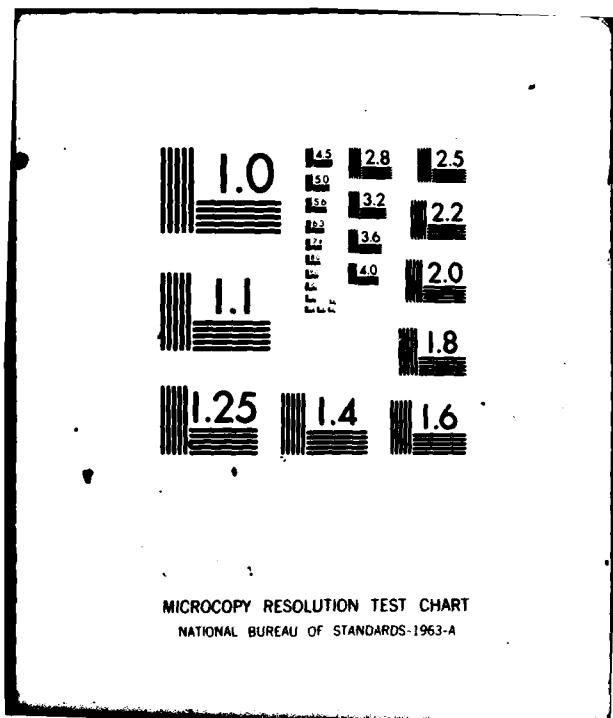
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## ROLE OF COMPLEMENT IN BLOOD PRESERVATION AND BLOOD BANKING

Annual Report

Author: *Yma O. Szymanski, M.D.*

January 1980

Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)		
<p>→ Experiments were undertaken to evaluate the role of complement in erythrocyte preservation. The major objective of the initial studies has been the development of a new immunochemical method to quantitate cell-bound C3. This method is based on the principles of antiglobulin (anti-C3) consumption, and it utilizes C3 sensitized zymosan as standards, the calibration of which is presently under study. -</p> <p>Since it was necessary to prepare complement-sensitized RBC in a reproducible</p>		

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manner for the quantitative cell-bound C3 assay, it became necessary to understand better the mechanism of complement uptake by RBC in low ionic strength solutions (LISS). In these studies, anti-D served as a tracer of immunoglobulin (Ig) reactions with Rh-negative RBC. The data showed that the major portion of anti-D consisting of Ig classes A and M and IgG subclasses 1 and 3 (the only ones detectable in anti-D) bound to RBC in LISS and eluted from RBC in normal ionic strength conditions. It appeared that the fixation of Ig to RBC initiated complement activation by the classical pathway. Bromelin modified RBC failed to support either Ig or complement binding, indicating that this treatment removed from RBC membrane material that formed the "antigen" site in LISS. The data showed also that EC4 and EC43 prepared in LISS using CPD plasma became nonspecifically sensitized with Ig, whereas E prepared in LISS using heated plasma did not, suggesting that Ig is involved in the initial assembly of complement components to cell membrane. The results impact on the low ionic crossmatch methods, providing an experimental model for further studies.

Our studies also focused on the quality control of anti-complement reagents. We prepared carefully characterized test cells which were glycerolized and stored frozen for prolonged periods. Following deglycerolization, the test cells could be stored at 4°C for at least two weeks. This approach improved the precision of quality control of antiglobulin sera and contributed positively to the cost-containment efforts in the blood bank.

ROLE OF COMPLEMENT IN BLOOD PRESERVATION AND BLOOD BANKING

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## SUMMARY

We report studies done during the contract period from May 1, 1979 to Dec. 31, 1979. Experiments were undertaken to evaluate the role of complement in erythrocyte preservation. The major objective of the initial studies has been the development and evaluation of a new method to quantitate cell-bound C3.

The new method utilizes the principles of antiglobulin (anti-C3) consumption. The amount of anti-C3 consumed by the unknown samples is compared to that consumed by known standards, and the anti-C3 consumption is measured in the AutoAnalyzer. We used zymosan-bound C3 as known standards. More C3b and C3d was found on stored RBC than on fresh RBC. We are now in the process of quantitating the amount of C3 on the zymosan standards by using radiolabeled, highly purified C3. Following the completion of this part of the study we can express the amount of cell-bound C3 in terms of number of molecules. C3b and C3d will be measured on both fresh and preserved RBC, and on erythrocytes of patients suffering from certain types of hemolytic anemias, on quality control cells, etc.

While developing the quantitative assay for cell-bound C3 measurement, it became necessary to understand better the mechanism of complement uptake by RBC in low ionic strength solutions (LISS). In these studies Rh-negative RBC and plasma containing anti-D antibodies were exposed to LISS at 37°C. Anti-D served as a tracer of immunoglobulin (Ig) behavior. The data showed that the major portion of anti-D consisting of Ig classes A and M and IgG subclasses 1 and 3 (the only ones detectable in anti-D) bound to RBC in LISS and that they eluted from RBC in normal ionic strength conditions. It appeared that the fixation of Ig to RBC initiated complement activation by the classical pathway. Bromelin modified RBC failed to support either Ig or complement binding in LISS, indicating that bromelin deleted membrane structures from RBC that formed the "antigen" site in LISS. The data showed also that EC4 and EC43 prepared in LISS using CPD plasma became nonspecifically sensitized with anti-Ig reactive material, and that E prepared in LISS using heated plasma did not. These data indicated that Ig is involved in the initial assembly of complement components to cell membrane, and impact on the low ionic crossmatch methods, providing an experimental model for further study.

Our studies also focused on the quality control of anti-complement reagents. We prepared carefully characterized test cells, which were glycerolized and stored frozen for prolonged periods. Following deglycerolization the test cells could be stored at 4°C for at least two weeks. This approach improved the precision of quality control of antiglobulin sera and contributed positively to the cost containment efforts in the blood bank.

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## STATEMENT OF THE PROBLEM

We have previously observed that fragments of the third component of human complement (in a state of either C3b or C3b') accumulate to RBC membrane during blood storage at 4°C. We also observed, as have others, that normal RBC have C3d molecules on their membrane.<sup>1,2,3,4</sup>

The studies done under this contract period (beginning May 1, 1979) were designed to develop a method to quantitate precisely cell-bound C3b and C3d. This is an integral part of the evaluation of the role of complement in RBC preservation.

Our method for quantitation of cell-bound C3 is novel, based on the principles of antiglobulin (anti-C3) consumption and utilizing the AutoAnalyzer to measure the strength of anti-C3 antisera. The strength of anti-C3 is proportional to its ability to agglutinate complement coated RBC in the AutoAnalyzer. EC43 prepared by the low ionic method was used as an indicator RBC in the AutoAnalyzer. The quantity of C3 bound to the unknown test cells is determined by measuring their ability to consume anti-C3, and comparing this effect to the anti-C3 consumption by known standards.

In order to standardize the test system, it was necessary to evaluate thoroughly the three reagents used in the test: anti-complement antibodies, EC43, and the C3 standard. Therefore, our work during this year has focused on three major areas:

1. Study of ways to prepare standards (Human cells or particles) having known amount of bound complement (C3), and performance of the assay test.
2. Study of the mechanism responsible for complement uptake by RBC in low ionic strength solutions (LISS), and characterization of the other related phenomena occurring under these conditions so that EC43 can be prepared in a reproducible manner.
3. Quality control of the anti-complement antisera by preparing test RBC that are coated only with single, specific complement components and storing them in frozen state for prolonged periods before use.

## BACKGROUND

Various methods exist for measurement of cell bound complement (C3).<sup>4-8</sup> Some of these techniques are based on anti-complement consumption techniques, as are ours, employing known amounts of soluble complement as known standards.<sup>5,6</sup> But since the curves characterizing anti-complement consumption by soluble and cell-bound standards differ, these methods are known to produce variable results. Other investigators have used RBC as standard, and quantitated the amount of complement added to the cell.<sup>8</sup> However, inaccuracies arise from the fact that normal, human RBC, e.g., are known to have cell-bound C3b. In our tests, therefore,

we tried to prepare known standards which would not have complement components attached to them in their native state so that we could quantitate the cell-bound C3 accurately.

The goal of our method was to measure relatively small amounts of cell-bound complement accurately. It was, therefore, of the utmost importance to develop a test that is sensitive and reproducible. Utilizing the AutoAnalyzer, the sensitivity of the agglutination of EC43 by anti-complement antibodies can be enhanced by increasing the quantity of RBC-bound C3b. It was also of importance to use test RBC which would not vary in the degree of sensitization from day to day. In the course of these studies we observed that when EC43 were prepared by exposure of RBC and plasma (or serum) to LISS, remarkable variability in the strength of C3 sensitization was observed from day to day. The results also varied depending on who was the plasma or RBC donor. It was our aim to prepare EC43 that were strongly sensitized with C3 and then store them in frozen state. Additional problems arose because heavily sensitized EC43 tolerated the glycerolization-freezing-deglycerolization procedure rather poorly. During this process, EC43 tended to lose the C3c component so that they became EC43d. Since the basic mechanism by which complement activation occurred in LISS had not been definitively clarified despite the availability of rather extensive literature on the topic, studies were initiated to clarify this question. Furthermore, the uptake of non-specific Ig or blood group antibodies by RBC in LISS is not fully understood. Since LISS played a key role in our test as well as in blood banking in general, the studies to be reported below are particularly relevant.

The concept whether anti-complement antibodies are important in blood banking compatibility tests have varied greatly during periods of time. At one time, need for anti-complement antibodies in broadspectrum antiglobulin sera was advocated with enthusiasm, whereas presently some blood banking authorities feel that they are totally unnecessary.<sup>10</sup> However, anti-C3d antibodies have a definite role in diagnosing complement sensitization of RBC in-vivo.<sup>10,11</sup> Although monovalent anti-C3d antibodies are commercially available, we have observed that they do not work as expected. It is, therefore, of utmost importance to test these and other antiglobulin antisera with rigorous quality control methods. This can be done by preparing RBC which are sensitized with single components of complement and immunoglobulin. Since their preparation can be time-consuming, many blood banks do not perform these procedures. We have utilized RBC freezing technics to glycerolize test cells so that they can be stored at -80°C for prolonged periods. These test cells can be deglycerolized at various periods of time and used as quality control cells making their preparation feasible in most blood banks. This part of the work will be also reported below.

## APPROACH TO THE PROBLEM

### I. Method to Quantitate RBC-bound C3 Preparation of EC43b

A unit of stored blood (21 days) was glycerolized by the Red Cross method.<sup>12</sup> The glycerolized blood was divided into several 10ml aliquots and stored frozen at -80°C. Each day of use an aliquot was thawed and deglycerolized. Sensitization of RBC with complement was as follows: One vol of 50% RBC in .9% NaCl was combined with one vol of pooled plasma (pool of 5, type A CPD plasmas, which had been heparinized and recalcified. Heparinizing and recalcifying was done by adding to one unit (about 250ml) of CPD plasma 2100 IU of heparin and 5ml of 10% calcium gluconate). Into this mixture was added 20 vol of 5% mannitol in H<sub>2</sub>O buffered to pH 6.0 with phosphate. The incubation took place at 37°C for 15 minutes. Following incubation, the agglomerated RBC were resuspended with .9% NaCl, pH 6.0, and washed three times with unbuffered .9% NaCl, whereafter they were suspended to Hct.=10 in .5% Ficoll in .9% NaCl.

### Preparation of Anti-complement Antisera

Anti-C3 (with specificity to the C3c fragment) was obtained from Atlantic Antibodies, Westbrook Me, and anti-C3 diluted 100,000 times caused about 80% agglutination of EC43 and was used as a target dilution. The target dilution of anti-C3d antibody was 1/5,000. Hetero-agglutinins were removed from these antisera as described below on page 8.

### Preparation of Standards

The definitive method to prepare particle-bound C3b standards cannot be fully described as yet. After experimenting with RBC-bound C3 standards, we found them too inaccurate since it was difficult to ascertain what was the quantity of RBC-bound C3b prior to sensitizing them with C3b. Therefore, we selected to use zymosan particles which will activate the complement system by the alternative pathway when added to serum. In order to quantitate accurately the amount of C3b bound to zymosan particles, we are using radiolabelled, highly purified C3 (obtained from two sources, Dr. H. Muller-Eberhard, La Jolla CA, and Dr. Brian Tack, Childrens Hospital Medical Center, Boston, MA). The radiolabeling is done by the lactoperoxidase method.<sup>13,14</sup> We have just now began a series of experiments testing our radiolabeling method. The standard will be prepared by adding the radiolabeled C3 to fresh, normal serum, and the mixture is incubated with zymosan particles (50mg zymosan for each ml of serum). The fraction of label found on washed zymosan particles will represent the proportion of total C3 (quantitated by radial immuno-diffusion method)<sup>15</sup> that was bound to zymosan.

### Performance of the Test

Various amounts of C3b-coated, washed zymosan particles were combined with target dilution of anti-C3 or anti-C3d. Dilutions of stored and fresh RBC as well as EC3b were also incubated with anti-C3 and anti-C3d. The incubation took place for 60 minutes at 37°C, following which the samples were centrifuged and the supernatants were tested for the remaining anti-C3 activity in the AutoAnalyzer.

## II. Study of the Mechanism of Complement and Ig Uptake by RBC in low ionic strength solutions (LISS)

### Blood Samples

From each of 51 volunteers (healthy laboratory personnel, medical students and hospital employees) between 10 to 20 ml of whole blood was collected into .13 vol of citrate-phosphate-dextrose anticoagulant (CPD, Fenwal Laboratories, Deerfield, IL) or with 1U of heparin. From some volunteers, blood samples were collected without anticoagulant for preparation of serum.

### Preparation of Serum and Plasma

CPD plasma, heparinized plasma and serum were separated from RBC by centrifugation. In some cases, plasma and serum were heat-inactivated by incubation at 56°C for 60 minutes. Following inactivation, the precipitated fibrin was removed by centrifugation and the clear supernatant was used for tests.

### Anti-Rh Antisera

Anti-Rh antisera were obtained from three sources: a) anti-D, lot R5822-3 (Ortho Diagnostics, Raritan, NJ), b) anti-CD "TUT", obtained from a blood donor, and c) anti-CD "CHA", also obtained from a blood donor.

### STUDIES OF COMPLEMENT UPTAKE BY RBC

We compared complement uptake by anti-A coated RBC and by normal RBC in LISS, when RBC were suspended either in serum, heated serum, or CPD plasma. Effect of bromelinization of RBC on complement uptake was also studied. Between five to ten experiments were done in each group.

### Preparation of EC43 by anti-A

Equal vols of washed, 5% RBC, type A<sub>2</sub>, and 1/16 dilution of commercial heat-inactivated anti-A, Lot A8605-2 (Ortho Diagnostics, Raritan, NJ) were combined. An equal vol of fresh type A serum was added and the mixture was incubated at 37°C for 15 minutes. Thereafter, the RBC were disaggregated by adding an equal vol of AB substance (Dade, Miami, FL). The disaggregated RBC were washed four times with .9% NaCl and tested for cell-bound complement components.

### Preparation of EC43 in LISS

The ionic strength of RBC-serum or -plasma mixtures was lowered by two different technical approaches. In the first one, 10 vol of phosphate buffered 5% mannitol, pH 6.0, was added to one vol of blood containing equal vols of 50% washed, packed RBC and serum (or plasma). This mixture was incubated at 37°C for 15 minutes, whereafter the packed RBC were disaggregated with phosphate buffered .9% NaCl, pH 6.0, and thereafter washed with nonbuffered .9% NaCl. In the second technique we combined one vol of 50% RBC with one vol of serum or plasma and dialyzed

it against at least 100 vol of phosphate buffered 5% mannitol, pH 6.0 at 37°C for 15 Minutes. Dialyzed RBCs and serum were separated by centrifugation. The separated serum of plasma was redialyzed against .9% NaCl to restore normal ionic strength. RBC were disaggregated with .9% unbuffered NaCl, the volume of which equalled the initial plasma volume.

#### Preparation of EC4 in LISS

The technique described above was used except that .2% K<sub>3</sub>EDTA was added to 5% mannitol.<sup>16</sup>

#### Bromelinization of RBC

Equal vols of 1% bromelin (Technicon Corp., Ardsley, NY) in .9% NaCl were added to 50% washed RBC in .9% NaCl. The mixture was incubated at 22°C for 15 minutes. Thereafter, RBC were washed three times and suspended in .9% NaCl.

#### Manual Direct Antiglobulin Tests

Manual direct antiglobulin tests were graded according to the recommendations of AABB.<sup>17</sup> In these tests anti-IgG, lot IGG112-1 (Gamma Biologicals, Inc., Houston, TX), and monospecific anti-complement sera were employed. The latter had been prepared for precipitating assays. Heteroagglutinins were removed from the anti-complement antisera the following was: One vol of five times diluted antiserum was incubated with .1 to .4 vol of specially prepared RBC at 22°C for 15 minutes. The preparation of RBC used for absorption was as follows: RBC were bromelinized (See above), washed three times with .9% NaCl and suspended as 50%. Equal vols of 50% bromelinized RBC and normal plasma were incubated at 22°C for 15 minutes. Thereafter, the RBC were washed four times with .9% nonbuffered NaCl and RBC were used for absorption as packed RBC. The use of bromelinized, plasma-treated RBC was necessary to remove the nonspecific component from the anti-complement antisera. The effectiveness of absorption was tested by preparing doubling dilutions from unabsorbed and absorbed sera and testing their ability to agglutinate

- a) normal, washed RBC
- b) bromelinized, plasma-treated RBC
- c) EC43 (see above)

Table 1 reports the results of these absorptions showing the reciprocal dilutions of antiglobulin sera that were reactive macroscopically with the test RBC. We used 80 times diluted absorbed anti-C4 and 10 times diluted absorbed anti-C3 for the manual antiglobulin tests. The specificity of these antisera were previously characterized: anti-C4 is directed against anti-C4c and anti-C3 against anti-C3c component.

#### STUDIES OF Ig UPTAKE BY RBC

The experimental protocol is shown in Figure 1. The experiments were designed to study the uptake of Ig from plasma to RBC in LISS and dissociation from RBC when ionic strength was normalized. In these tests, RBC and plasma were exposed to LISS by dialysis. In most of

Figure 1

Experimental protocol to study Ig uptake by RBC in LISS.

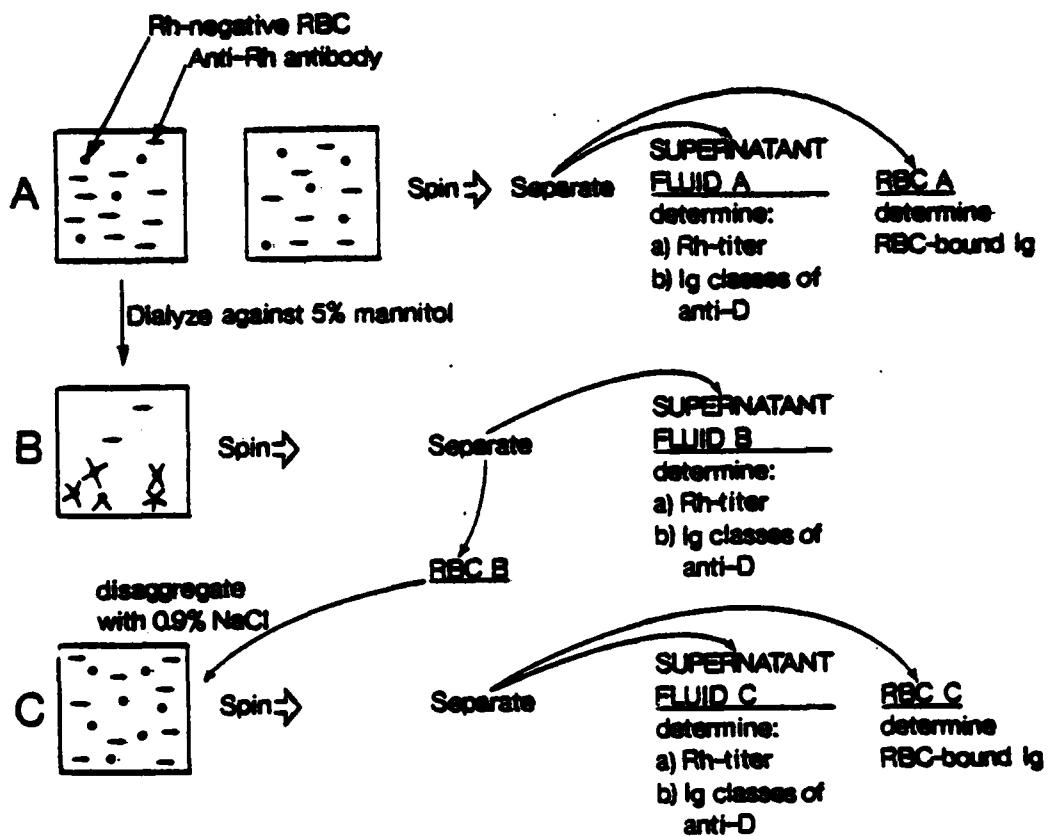


Table 1

## Effect of heteroagglutinin removal from the anti-complement antisera

Specificity	Manufacturer	Reciprocal dilution of antiserum causing agglutination of test RBC							
		E (normal)		plasma-treated)		E (bromelin,		EC43	
	Lot No.	pre*	post*	pre	post	pre	post	pre	post
Anti-C4 (anti-C4c)	Atlantic Antibodies C4 P016N	10	N.R.**	160	N.R.	2560	1280		
Anti-C3 (anti-C3c)	Atlantic Antibodies C3 P025N		N.R.	160	N.R.	1280	1280		

\*pre and post removal of heteroagglutinins by absorption

\*\*non-reactive

these studies, we employed Rh-negative RBC and anti-Rh antibodies, which served as tracer for all Ig.

#### Studies of anti-D in the supernatant

The anti-D in the supernatant fluids A, D and C was assayed the following way:

a) Rh titer was done by the standard technique using doubling dilutions of the antisera.<sup>1</sup> The endpoint of the titer was the reciprocal of the highest dilution causing macroscopic agglutination of RBC. For comparison, uptake of Rh-antibody by Rh-positive RBC was also studied. In these studies, a vol of 50% Rh-positive RBC was incubated for 30 minutes at 37°C with equal vol of plasma containing the same amount of Rh antibodies as in the LISS method. The titer of the antibodies remaining in the supernatant after absorption was determined. Rh antibodies were eluted from the sensitized RBC by the modified acid elution technique<sup>18</sup> and the titer was determined. The volume of the eluate was the same as the initial plasma volume.

b) Semiquantitative assay of Ig classes and IgG subclasses of the Rh-antibodies was performed using AutoAnalyzer. In these studies, a vol of pooled supernatant A, B and C (Fig. 1) was each incubated with five vol of Rh-positive RBC at 37°C for 30 minutes. Thereafter, the RBC were washed four times with .9% NaCl and suspended as 20% in .5% ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) and .9% NaCl. The RBC were tested before and after sensitization in the AutoAnalyzer (Technicon Corp., Ardsley, NY) for membrane-bound Ig. This method of assay has been described previously.<sup>1,19,20</sup> Briefly, in this test 20% RBC and dilutions of anti-immunoglobulin antisera in .9% PVP-K-90 (Technicon Corp., Ardsley, NY) were introduced into the Autoanalyzer under continuous flow conditions and incubated for about 30 minutes at 22°C. Thereafter, the agglutinated RBC were removed by special T-fittings and the remaining RBC were hemolyzed with 1% Triton (Fisher Scientific Co., Pittsburgh, PA) in distilled water. The optical density of the hemolysate was measured colorimetrically. The degree of agglutination was quantitated by determining the percent agglutination as follows:

$$\text{Percent agglutination} = 100 \times \frac{\text{O.D. without Ab} - \text{O.D. with Ab}}{\text{O.D. without Ab}}$$

The anti-Ig antisera were used in dilutions which agglutinated normal RBC only slightly or not at all. The diluted anti-IgA, however, agglutinated RBC more strongly than other antibodies. The agglutination value obtained with nonsensitized RBC was subtracted from that obtained with anti-D sensitized RBC to determine the contribution of bound anti-D to these reactions. The result was called "A percent agglutination".

#### Anti-Ig Antisera

Heteroagglutinins were removed from all anti-Ig antisera by absorption with well-washed normal RBC (mixture of types A, B and O). After absorption the antisera failed to agglutinate normal RBC in manual tests and bromelintreated RBC in the automated tests.<sup>2</sup> Table 2 shows the source of each antiserum used, and the dilution employed, as well as the

Table 2

Characteristics of the anti-Ig antisera used in the automated antiglobulin test

<u>Specificity of the anti-Ig serum</u>	<u>Manufacturer</u>	<u>Lot No.</u>	<u>Dilution of antiserum used</u>	<u>Agglutination of normal RBC, % Mean <math>\pm</math> S.D. (n=14)</u>
G (broad spectrum, Fc-fragment specific)	Atlantic Antibodies	IgG P049N	1/300,000	10.9 $\pm$ 5.4
G1	Dutch Red Cross			
	KH 161 01 P2		1/5,000	4.6 $\pm$ 3.3
G2	Dutch Red Cross			
	SH 162 06 P1		1/1,000	9.1 $\pm$ 4.3
G3	Dutch Red Cross			
	KH 163 41 P1		1/200	0.07 $\pm$ 0.47
G4	Dutch Red Cross			
	SH 64 04 P2		1/1,000	2.8 $\pm$ 3.5
A	Atlantic Antibodies			
	IgA P045N		1/5,000	21.2 $\pm$ 9.9
M	Atlantic Antibodies			
	KH 4214 P04		1/200	5.36 $\pm$ 3.36

average agglutination value observed with normal RBC. The specificities of anti-IgA, anti-IgM and of the anti-IgG subclass antisera were studied by coating RBC with various blood group antibodies.<sup>21</sup> Some IgG antibodies elicited agglutination only with anti-IgG1, others with anti-IgG3, or with IgG4, and some with both anti-IgG2, and anti-IgG4, indicating that these antibodies did not crossreact with each other. Only rare blood group antibody had IgA components, and some had IgM component in addition to IgG component.

#### Studies on Ig bound to disaggregated RBC

The permanent fixation of Ig to membrane of RBC that had undergone aggregation in LISS and then disaggregated, was determined by the Auto-Analyzer techniques as described above. The results were expressed as  $\Delta$  percent agglutination.

Total of eight studies were done by exposing RBC and CPD plasma to LISS so that EC43 was produced. In four of eight cases we used anti-Rh antibodies and Rh-negative RBC. In the remaining four cases we used autologous CPD plasma without added antibody.

Total of two studies were done by exposing RBC and autologous plasma to LISS in the presence of .2% K<sub>3</sub>EDTA, so that EC4 was produced.

Total of six studies were done by exposing RBC and heated CPD plasma to LISS either in the presence of absence of Rh-antibodies. These RBC did not become complement coated.

### III. Quality control of the anti-complement and anti-Ig antisera by using frozen-preserved test RBC.

#### PREPARATION ON TEST CELLS

##### RBC Sensitized with IgG

Commercial anti-D (Ortho Diagnostics, Raritan, NJ) was used to sensitize RBC with IgG and anti-D. The sensitized RBC had 1+ positive manual direct antiglobulin test. The vol of antiserum to be used per one vol of RBC was determined by aide of anti-D titer. We incubated a 10ml aliquot of once washed, packed, type O RBC with 1ml of anti-D at 37°C for 30 minutes. The sensitized RBC were washed once prior to freezing.

##### RBC Sensitized with Complement Components

EC43 were prepared by the low ionic strength procedure.<sup>22</sup> One vol packed RBC were combined with 1.5 vol of serum, or with either heparinized or CPD plasma. Twenty vol of 5% mannitol in phosphate buffer, pH 6.0, were added and the mixture was incubated at 37°C for about 15 minutes. The aggregated RBC were suspended in .9% NaCl, pH 6.0, and washed once prior to the freezing procedure.

EC4b were prepared as above, but the volumetric ratio between plasma and RBC was 3 and the 5% mannitol contained .2% K<sub>3</sub>EDTA.<sup>16,23</sup>

EC3b were prepared according to the method of Fruitstone.<sup>24</sup>

EC3d were prepared by the method of Chaplin et al, which is a modification of the Fruitstone method.<sup>17</sup> This method resulted in stronger sensitization of RBC with C3d than is the case with the Fruitstone method.

EC3d were also obtained from a patient suffering from *Mycoplasma Pneumoniae* infection. This patient had cold agglutinin titers of 1/4000 with adult O RBC and his RBC gave positive manual AGT only with anti-C3d, and negative with other antiglobulin sera.

#### Glycerolization of test RBC

Two vol of 6.2M glycerol containing 0.3g KCl/dl, 1.6g sodium lactate/dl, pH about 7.4 (Cytosol Labs, Inc., Boston, MA) was added to a vol of sensitized RBC (Hct. approximately 90%) in two steps. One-fifth of the glycerol volume was added to RBC dropwise, mixing the sample in Vortex mixer. The remaining glycerol was added about ten minutes later, introducing it into RBC gradually and under constant mixing. Aliquots containing about 0.5ml glycerolized blood were stored frozen at -80°C.

#### Deglycerolization of test RBC

The aliquots were thawed in 37°C waterbath. One-quarter vol of 12% NaCl, pH between 6.7 to 7.4 (Cytosol Labs Inc., Boston, MA) was added to a vol of glycerolized, thawed RBC dropwise under constant mixing. Following a period of 2 minutes or more, a vol (equal to that of the thawed sample) of 1.6% NaCl, pH about 6.7 to 7.4, was added as described before. The sample was centrifuged following a period of two minutes or more using 2500 g for four minutes. Following the removal of the supernatant fluid, the RBC were washed 3 times with a vol (equal to that of the thawed sample) of 1.6% NaCl, whereafter they were washed with .9% NaCl until the supernatant was clear. The deglycerolized RBC were stored at 4°C as 5% suspension in .9% NaCl.

#### The percentage Recovery of test RBC after Freezing and Deglycerolization

The percentage recovery of test RBC was measured by determining the volume and hemoglobin concentration of both the thawed glycerolized blood and of the deglycerolized samples, and the total Hb in the deglycerolized sample was divided with that in the thawed sample.<sup>25</sup> Hemoglobin concentration was determined by cyanmethemoglobin method.<sup>17</sup> One percent Triton X-100 was added into the cyanmethemoglobin reagent to facilitate hemolysis of the glycerolized blood.

#### Antiglobulin Tests

Antiglobulin tests were done by the conventional way as described in the Technical Manual of the American Association of Blood Banks, and the degree of agglutination was graded as described in that manual.<sup>17</sup> The antiglobulin tests were performed on the prepared test RBC before glycerolization and after glycerolization, freezing and deglycerolization. These tests were done also following periods of storage at 4°C for up to two weeks.

### Antiglobulin Sera

Anti-IgG (Ortho Diagnostics, Raritan, NJ) was used according to the manufacturer's directions.

### Anti-Complement Antisera

We utilized monospecific anti-complement antisera prepared for precipitating assays. Heteroagglutinins were removed the following way: One vol of five times diluted antiserum was absorbed with .1 vol of packed RBC which consisted of a mixture of trypsinized RBC (types A, B and O). The trypsinization was carried out by mixing four vol of 0.1% Trypsin (Difco Laboratories) to one vol of packed RBC and the mixture was incubated at 37°C for 20 minutes. Thereafter, RBC were washed 4 times with large vol of 0.9% NaCl. Following absorption, the antisera did not agglutinate either normal or trypsinized RBC, but agglutinated EC43 when diluted over thousand-fold.

Anti-C3 (Atlantic Antibodies, Westbrook, ME), Lot No. C3 P025N, contained only the anti-C3c antibody as previously described.<sup>1</sup> The antiserum was diluted 10 times for use.

Anti-C3d (Dutch Red Cross), was used in dilution 1/40.

Anti-C4 (Atlantic Antibodies, Westbrook, ME), Lot No. C4 P016N, contained only C4c activity. It was used in dilution 1/80.

## RESULTS

### I. Method to Quantitate RBC-bound C3

Figure 2 shows the percent neutralization of anti-C3 and anti-C3d antibodies by C3b coated zymosan particles. These data show that zymosan standards can be stored in frozen state.

Figure 3 shows the percent neutralization of anti-C3 by fresh and stored RBC, and by EC43b, EC43b appeared to contain about 20 times more C3b per RBC than stored RBC.

Figure 4 shows the percent neutralization of anti-C3d by both fresh and stored RBC. More anti-C3d was consumed by the stored RBC than by fresh RBC, although the agglutination of stored RBC with anti-C3d was less than that with fresh RBC. The discrepancy between the results of the agglutination and neutralization tests indicate that there is a loss of RBC agglutinability during blood storage at 4°C.

### II. Study of the Mechanism of Complement and Ig Uptake by RBC in LISS Studies on Complement Uptake by RBC

Table 3 shows the comparison of complement uptake by RBC aggregated in LISS and by RBC agglutinated with anti-A. When either heparinized plasma or serum was used as a source of complement, both the aggregated RBC and anti-A coated RBC became heavily sensitized with C4 and C3. Although not shown in Table 3, we confirmed the previously reported

Figure 2

Neutralization of anti-C3 and anti-C3d antibodies with C3b-coated zymosan particles.

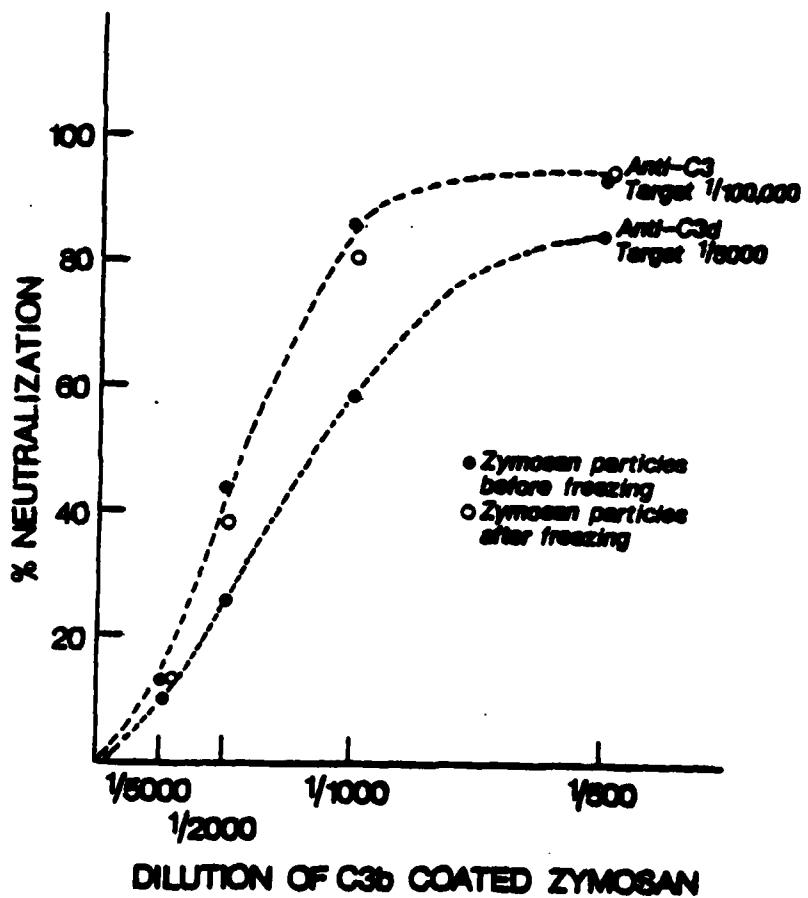


Figure 3

Neutralization of anti-C3c (1/100,000 target) antibody with fresh, stored, and LISS RBCs.

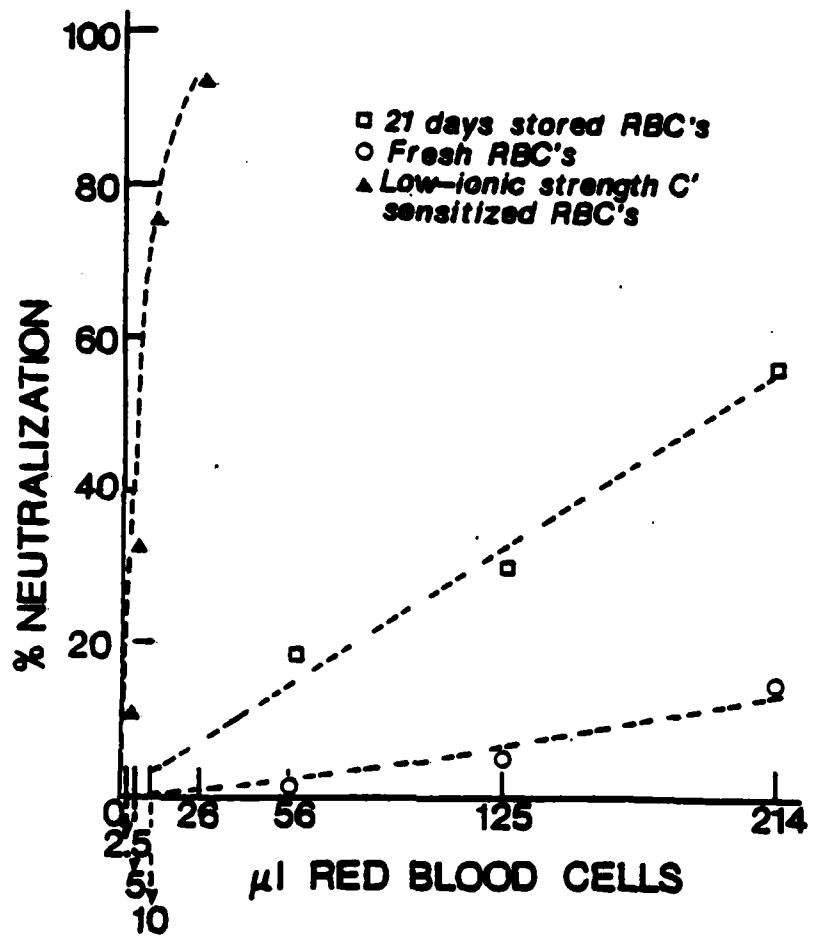


Figure 4

Neutralization of anti-C3d, 1/5000 target, with fresh and stored RBCs.

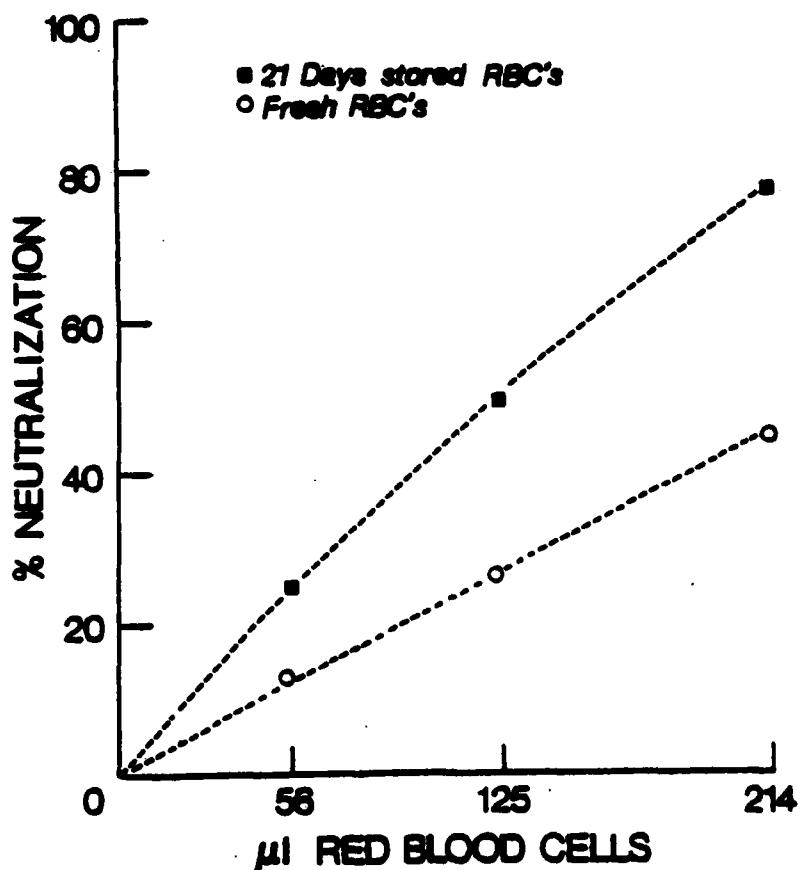


Table 3

Comparison of complement uptake by a) RBC aggregated in LISS and by b) RBC agglutinated with anti-A. The RBC-bound Ig were removed by normalization of ionic strength in the former case and by use of soluble A substance in the latter case.

Source of Complement	Pre Treatment of RBC	a) RBC in LISS			b) RBC + anti-A		
		-IgG	-C4	-C3	-IgG	-C4	-C3
Serum or heparin plasma	washed	0	4+	4+	0	3+	3+
-11-	bramelin modified	0	0	0	0	4+	4+
Heated serum	washed	0	0	0	0	0	0

finding that anticoagulated plasma supported complement uptake by RBC aggregated in LISS, but not by RBC coated with anti-A.<sup>26</sup> The bromelin modified RBC did not aggregate nor fix complement in LISS but they bound both anti-A and complement avidly. Inactivated serum, however, did not support complement activation in either system.

### Studies on Ig Uptake by RBC

#### Supernatant anti-Rh-titers

Table 4 shows the titers of the marker antibody in the supernatant before, during and after exposure to LISS. Comparison of these data to the anti-Rh antibody uptake by Rh-positive RBC in normal ionic conditions revealed that on the average, slightly less antibody was removed from plasma by the Rh-negative RBC in LISS. Most of the original anti-Rh antibody was recovered in the supernatant fluid by normalizing ionic strength; in contrast, significantly smaller proportion of antibodies were recovered by the acid elution method.

#### Immunoglobulin Classes of the Supernatant anti-Rh Antibodies

Figure 5 shows the results of a semiquantitative assay of Ig classes and subclasses of anti-D antibody present in the supernatant before, during and after the exposure of Rh-negative RBC and anti-D to LISS. To obtain these data, supernatants A, B, and C of three separate experiments were combined, and Rh-positive RBC were sensitized with pools A, B, and C. Since the anti-D did not contain detectable amounts of IgG2 or subclasses, the behavior of IgG1 and IgG3 only could be studied. These data show that reduction of the ionic strength of the anticoagulated blood samples caused adherence of anti-D consisting of IgG1, IgG3, IgA, and IgM to the membrane of Rh-negative RBC, and that most of the initially available IgG1 and IgM anti-D was recovered in the supernatant fluid when the ionic strength was normalized, whereas proportionally less of the initial IgA and IgG3 anti-D appeared to be recovered in the eluate.

#### Ig Bound to the Disaggregated RBC

RBC which had been suspended in CPD plasma, exposed to LISS and subsequently disaggregated with 0.9% NaCl, became EC43 and also agglutinated more strongly with anti-Ig antisera than they did before the procedure, indicating that some Ig remained RBC-bound. The increase of anti-Ig induced agglutination was unaffected by anti-Rh antibodies that were added to Rh-negative RBC, since similar increases were observed when autologous plasma was employed ( $p > .2$ ). RBC suspended in CPD plasma and exposed to LISS in the presence of .2% K<sub>2</sub>EDTA and then disaggregated with 0.9% NaCl became sensitized with C4 and reacted similarly with anti-Ig antisera in the AutoAnalyzer as did EC43.

RBC which had been suspended in heated CPD plasma, exposed to LISS and then disaggregated with 0.9% NaCl did not become sensitized with complement, nor did they agglutinate with anti-Ig antisera more than they did before the procedure. The data are presented in Figure 6. Comparison between EC43/EC4 and E revealed significant difference in

Table 4

Titer of the anti-Rh antibody before, during and after exposure of Rh-negative RBC and anti-D antibody to LISS. Comparison of the antibody titers following absorption-elution procedure with Rh-positive RBC are also shown.

<u>ANTIBODY</u>	<u>SOURCE</u>	<u>BEFORE</u>	<u>TITER</u>	<u>DURING</u>	<u>AFTER</u>
$\bar{X}^*$ (range)					
<u>LISS PROCEDURE, USING RH-NEG RBC</u>					
ANTI-CD	DONOR, CHA N**=6	114 (64-128)	18 (8-64)	102 (64-128)	
ANTI-CU	DONOR, TUT N=2	2048 (2048)	128 (128)	1024 (1024)	
ANTI-D DIL. 1/3	ORTHO LOT R536 N=8	512 (512)	54 (16-128)	395 (256-512)	
<u>NORMAL IONIC PROCEDURE, USING RH-POS RBC</u>					
ANTI-D DIL. 1/3	ORTHO LOT R536 N=5	512 (512)	37 (32-64)	128 (128)	

$\bar{X}^*$  = mean

\*\*N = number of experiments

Fig. 5

Agglutination of anti-D sensitized RBC with various anti-Ig antisera. Five vol of packed, type O, Rh-positive RBC were sensitized with one vol of plasma containing anti-D. The plasma had been previously mixed with Rh-negative RBC and samples were collected before, during and after exposure of the mixture to LISS. Supernatants of three experiments were pooled and results are shown in the graph.

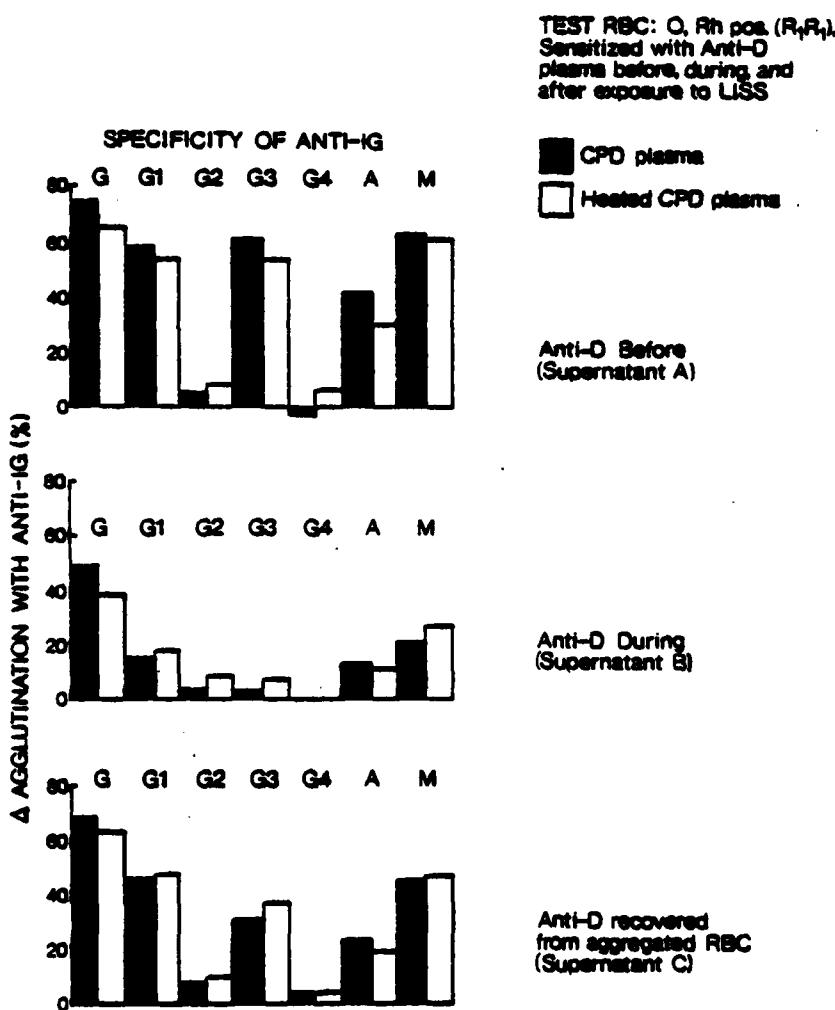
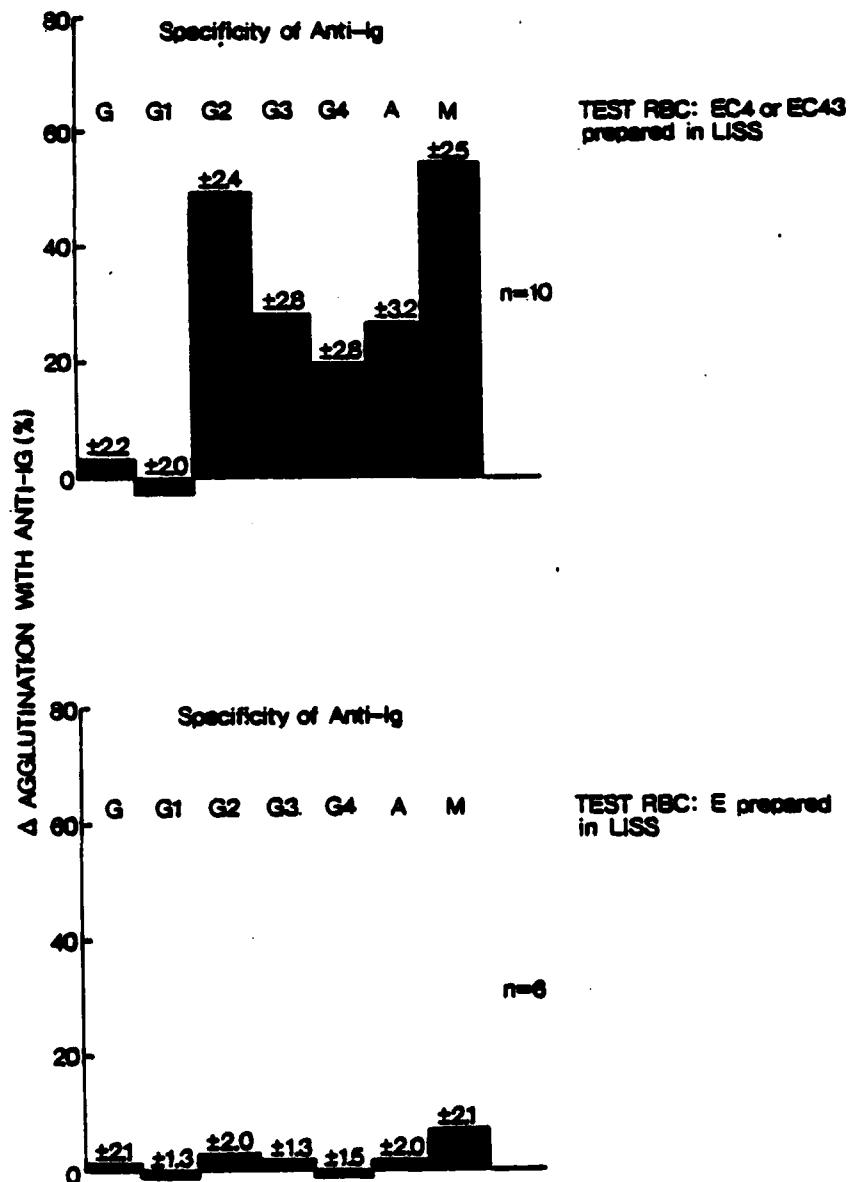


Fig. 6

Anti-Ig induced agglutination of EC4,3/EC4 and E prepared by exposure to LISS. Mean  $\pm$  S.E. of agglutination are shown. Significant difference in agglutination was observed between EC4,3/EC4 and E when anti-IgG2, anti-IgG3, anti-IgG4, anti-IgA or anti-IgM was used ( $p < .002$  in all cases.)



agglutination when we used antiIgG2 ( $t=15.04$ ,  $p<0.002$ ), anti-IgG3 ( $t=8.78$ ,  $p<0.002$ ), anti-IgG4 ( $t=6.36$ ,  $p<0.002$ ), anti-IgA ( $t=6.68$ ,  $p<0.002$ ) and anti-IgM ( $t=14.85$ ,  $p<0.002$ ). These results indicate that all classes of Ig except IgG1 were fixed to EC43 and EC4. Since the results using anti-IgG (Broadspectrum) were similar to those obtained with anti-IgG1, it appears that the activity in the anti-IgG under our test conditions was mainly directed to the IgG1 component (used in dilution 1/300,000).

### III. Quality Control of the Anti-complement and Anti-Ig Antisera by Using Frozen Preserved test RBC

The total recovery of test cells following glycerolization, freezing and deglycerolization was  $82.8 \pm 9.5\%$  (mean  $\pm$  S.D.)  $n=12$ .

Preparation of EC43 by the low ionic method consistently produced test RBC which were strongly sensitized with C4 and moderately strongly sensitized with C3. Following glycerolization, freezing and deglycerolization, the test cells tended to lose their ability to agglutinate with anti-C3, but their ability to agglutinate with anti-C3d remained unchanged. These findings indicated that the C3c fragment was lost from RBC-bound C3b during the procedure. We were unable to determine the exact cause of this phenomenon. When nonstored RBC were used to prepare EC43, they tended to lose their anti-C3c reactivity following deglycerolization more often than EC43 prepared using stored RBC. This finding, however, was not always observed. We also studied whether prolonged exposure of thawed EC43 to 12% NaCl affected their reactivity with anti-C3c. This was determined not to be the case.

Preparation of EC4 with the low ionic method and using K<sub>2</sub>EDTA produced RBC which were strongly coated with only C4; EC3 prepared with the method of Fruitstone<sup>27</sup> reacted weakly, 1+, with both anti-C3 and anti-C3d. EC3b prepared by the method of Chaplin et al., who used a modification of Fruitstone method, produced EC3b that reacted more strongly with both broadspectrum antiglobulin reagent and with monospecific anti-C3d (2+).

Table 5 shows the reactivity of various test cells with a battery of antiglobulin sera following glycerolization, freezing, storage at -80°C, deglycerolization and subsequent storage at 4°C for up to two weeks. During this period, there was no discernible loss of reactivity of E1g with anti-IgG. Reactivity of both EC4 and EC43 with anti-C4 declined from 4+ to 2+ during the two week post-thaw period. In addition, there was an abrupt loss of reactivity of EC43 and EC3 with anti-C3 during the post-thaw storage period. However, these test RBC remained strongly reactive with anti-C3d.

**Table 5**  
**Effect of the length of storage at 4°C after deglycerolization on test RBC reactivity**  
**(manual direct antiglobulin test).**

		Length of Storage at 4°C														
		0-1 day	1 week	2 weeks												
		BS* C4 C3c C3d IgG	BS* C4 C3c C3d IgG	BS* C4 C3c C3d IgG	BS* C4 C3c C3d IgG	BS* C4 C3c C3d IgG	BS* C4 C3c C3d IgG									
T	E43	2+	4+	0-2+	2+	0	2+	3+	0	2+	0	2+	0	2+	0	
E	EC3d, Ap**	±	0	0	2+	0	±	0	0	1+	0	±	0	0	±	0
S	EC3d***	1+	0	0	2+	0	1+	0	0	2+	0	1+	0	0	2+	0
T																
R	EC4	2+	4+	0	0	0	2+	3+	0	0	0	1+	2+	0	0	0
B																
C	E19	0	0	0	0	1+	0	0	0	0	1+	0	0	0	0	1+

BS\* broadspectrum antiglobulin reagent (Ortho Diagnostics, Raritan, NJ)

EC3d, Ap\*\* complement activation initiated by the alternative pathway

EC3d\*\*\* complement activation initiated by the classical pathway; RBC probably sensitized also with C4d

## DISCUSSION

### I. The method to Quantitate RBC-bound C3

Although a complete discussion of the method to quantitate cell-bound C3 and C3d and its applications is not possible as yet, the data shown above prove that the method is simple, sensitive, and accurate. Utilization of particle-bound C3b standards offers a significant advantage over RBC-bound C3 standards.

The new method will be applied to the quantitation of complement bound to stored and fresh RBC, to quality control RBC, to RBC of patients suffering from certain kinds of hemolytic anemias, etc.

### II. Study of the Mechanism of Complement and Ig Uptake by RBC in Low Ionic Strength Solutions

In the Classical pathway, complement sequence is activated by antibody coated cells, e.g., by RBC-bound anti-A. In this process, Clq binds to Fc fragments of IgG and IgM.<sup>27</sup> Different IgG subclasses have variable affinities to Clq. G3 has the highest affinity, followed by G1, G2 and G4.<sup>28</sup> If Clq binding leads to activation, changes occur in the sub-components of Cl and C1s acquires enzymatic activity. These reactions are Ca<sup>++</sup> dependent. The active C1s removes activation peptides from C4 and C2. Fusion occurs between the major portions of C4 and C2 in the presence of Mg<sup>++</sup>,<sup>29</sup> and through a site in the C4 portion the complex may attach to RBC occupying a membrane site which is close to but different from the IgG binding site.<sup>28</sup> The active C42 complex splits C3 into two separate components, C3a, the anaphylatoxin, and C3b, which may bind to RBC. When anti-A is eluted from RBC by the use of soluble A substance, both C4 and C3 remain RBC-bound. The present study was carried out to determine whether the complement activation in the LISS fits this model.

Since heating plasma to 56°C destroys complement components Clq, Clr, and C2,<sup>30</sup> anti-A coated RBC and those aggregated in LISS do not fix complement in the presence of heated serum. Anti-A sensitized RBC did not fix complement when suspended in citrated plasma, whereas RBC aggregated in LISS fixed both C3 and C4. This difference has been previously investigated and is considered to be related to differences in Ca and Mg ionization under normal and low ionic strength conditions.<sup>16</sup> Brumelin modified RBC bound anti-A and complement, but they did not aggregate nor bind complement in LISS. These findings indicate that the proteolytic enzyme brumelin removed membrane structures which formed the Ig binding sites during LISS exposure.

Since RBC aggregation in LISS does not involve specific antigen-antibody reactions, characterization of these "antibodies" has been incomplete. We investigated these antibodies using anti-D as Ig marker while employing Rh-negative RBC. Both manual and automated studies with the marker anti-D revealed that the major fraction of anti-D was bound to RBC during aggregation and released from RBC in normal ionic strength conditions. The binding of the "antibody" was independent of complement fixation. Comparison of Ig classes and IgG subclasses of the anti-D

before the procedure to those recovered from the aggregated RBC revealed that proportionally more IgG1 was recovered than IgG3. The recovery of IgM also appeared to be more complete than that of IgA. Moreover, the automated data showed that the absence of active complement during low ionic strength procedure did not decrease the recovery of anti-D in the final eluate. On the basis of these studies it appears that normal Ig behaved as "antibodies" to RBC in LISS. Formation of membrane antigen site in LISS caused antigen-antibody like reactions to occur on RBC membrane resulting in complement activation by the classical pathway.

Studies on the membrane-bound Ig revealed that some Ig remained on disaggregated EC43 and EC4, whereas no residual Ig were detected on RBC which had not become complement sensitized. It is of interest that in their original report Mollison and Polley noted that EC43 reacted weakly with anti-Ig.<sup>31</sup> We detected Ig classes G, A and M as well as IgG subclasses 2,3, and 4 on EC4 and EC43 when RBC and plasma were exposed to LISS. It was noteworthy that EC43 gave consistently negative manual antiglobulin tests with anti-IgG, and that the sensitive automated anti-globulin test remained negative with the broadspectrum anti-IgG antibody in the automated test. These findings indicated that the specificity of the diluted anti-IgG antibody was mainly directed against the IgG1 subclass. Since undiluted anti-IgG was used in the manual test, it is possible that if it contains antibodies to other IgG subclasses, the amount of Ig on EC43 was below the level of detection. The presence of Ig on EC4 and EC43 but not on E (produced by the low ionic strength procedure) was of interest. Goers and Porter reported that during complement activation by the classical pathway, C4 molecules bind to cell-bound Ig, probably to the Fab-portion.<sup>32</sup> They suggest that only those C4 molecules which bind to Ig are active in complement sequence. Furthermore, they show that the active C4 had to bind to Ig-C1q complexes or near this site. Although our findings are compatible with the above interpretation, they also indicate that C4 had binding sites to both Ig and RBC membrane. However, further studies are required to clarify this phenomenon. We do not know why IgG1 was not involved since it has a relatively high affinity to C1q.

Since LISS enhance both the rate and effectiveness of antibody uptake by RBC, these conditions have been incorporated to both manual and automated antibody detection tests.<sup>33-36</sup> The LISS crossmatch is now used in many blood banks. Our findings impact on these tests. Since low ionic strength tests resulted in binding of both C4 and Ig to RBC, this phenomenon could under certain circumstances result in nonspecific, positive manual AGT even when using anti-IgG reagent. The positive AGT could occur on the basis of quantitative variation of IgG subclasses in human sera, and when antiglobulin sera contain high concentrations of selected anti-IgG subclass antibodies. The high level of anti-IgG3 is particularly desirable in the antiglobulin sera, since blood group antibodies of the IgG3 subclass cause accelerated RBC destruction and if undetected may result in transfusion reaction.<sup>37</sup> The nonspecific nature of the positive AGT can, however, be verified by repeating the tests with heated plasma. Negative reactions should be observed under these conditions.

It is of interest, that Atchley et al reported that anti-D uptake by D-positive RBC in LISS could not be increased by enzyme modification of RBC, although LISS enhanced the uptake of anti-D of enzyme modified RBC.<sup>35</sup> Considering that bromelin modified RBC failed to fix nonspecific Ig, it is possible that LISS enhance the uptake of specific antibodies differently than nonspecific Ig.

It should also be noted that the recovery of antibodies using specific absorption-elution methods was less effective than that by the low ionic strength method using antigen-negative RBC. The latter approach could be exploited for purposes of antibody isolation and purification on the blood bank level.

### III. Quality Control of the anti-complement and Anti-Ig Antisera by Using Frozen Preserved Test RBC

Manual antiglobulin tests are used to detect RBC sensitization by either immunoglobulins or by complement components. Depending on the specificity and potency of the antiglobulin sera, it is possible to determine the class and subclass of the RBC-bound immunoglobulins as well as the fragment of the complement component causing RBC sensitization. In order to determine the specificities of antiglobulin sera that are most advantageously used in blood bank, it is necessary to evaluate under which clinical conditions various Ig and complement components can sensitize RBC in-vivo and cause their accelerated destruction, and whether pre-transfusion compatibility tests can be performed utilizing either anti-Ig reagents only or with association of anticomplement reagents.

Currently, many blood banks employ LISS to enhance the rate and quantity of antibody uptake by RBC.<sup>36,38</sup> These methods appear to possess a higher sensitivity than the normal ionic methods. Since complement uptake occurs also when ionic strength is reduced to 20% of normal, only anti-Ig antisera are usually employed. Furthermore, it has been questioned whether anti-complement activity is necessary in antiglobulin sera used to perform pre-transfusion compatibility tests in normal ionic conditions.<sup>10</sup> Indeed, complement uptake due to presence of cold agglutinins or for some other reasons may result in nonspecific positive AGT creating unnecessary delay of transfusions. On the other hand, it has been reported that some antibodies, although rare, can be detected only by their ability to bind complement.<sup>11</sup> Until this issue is satisfactorily settled, blood banks have the option of using antiglobulin sera that contain anti-Ig either alone or in combination with anti-complement antisera.

When AGT are used to characterize autoimmune hemolytic anemias, both anti-Ig and anti-complement antisera have an important role. As reported previously RBC of patients suffering from immune hemolytic anemia can be sensitized either with Ig alone, with Ig and complement, or with complement alone.<sup>10</sup> It is noteworthy that due to the presence of C3b inactivator and its cofactor in normal sera, the most important complement fragment on RBC in vivo is C3d, not C3b.<sup>11,22</sup> Therefore, monospecific anti-C3d antisera must be used to diagnose hemolytic anemias characterized by RBC sensitization with complement.

Although it appears that employment of monospecific anti-Ig and anti-C3d would be the major antisera needed to perform most in-vivo and in-vitro testing in blood banks, it might be desirable to use other anti-sera such as anti-C4 to explore its usefulness at least on experimental level. Regardless of the importance of the anti-C3d antisera, we have observed that the potency of the commercial anti-C3d antibodies is very weak and sometimes nonexisting.

Quality control of the antiglobulin sera, therefore, is a daily necessity in the blood bank. Depending on the specificity of the anti-globulin sera used in a particular blood bank, various types of quality control RBC may be used. On the basis of the previous discussion, the two most useful test cells are EIgG and EC3d. It might be useful to employ EC4 also. Although methods to prepare EC3b have been published,<sup>22,24</sup> they tend to produce RBC that are very weakly coated with C3b. The new method of Chaplin et al., which utilized Fruitstone method with added Mg in the reaction medium, produces RBC that are strongly coated with C3b. These RBC can be easily converted to EC3d by trypsinization. We have also used EC3d that were obtained from a patient suffering from *Mycoplasma Pneumoniae* infection and who had elevated cold agglutinin titers. These RBC were non-reactive with anti-C4, probably because C4d was present on cell membrane and the specificity of our antiserum was directed against C4c. We have also evaluated EC43 as test cells since they are simple to prepare. The glycerolization-freezing-deglycerolization procedure did not affect RBC-complement or RBC-IgG bond, nor was there loss of C4 from RBC during the procedure. Although there was no loss of C3d from RBC during this procedure, the C3c Fragment tended to be lost from the deglycerolized test cells. In those cases where the test cells retained their anti-C3c reactivity during the deglycerolization procedure, they tended to lose it during the post-thaw storage. There was also progressive loss of the anti-C4 reactivity of the appropriate test cells, so that EC4 that reacted initially 4+, reacted 2+ after two weeks of storage at 4°C. The reactivity of the appropriate test RBC with anti-C3d remained good during the two weeks post-thaw period.

Although preparation of well-characterized test cells can be time-consuming, the ability to freeze them for extended periods of time makes their preparation in blood banks practical. It is necessary to prepare only a small quantity of test cells (between 5 to 10 ml packed cells), glycerolize them, and freeze them in small aliquots. A 10ml vol of packed test cells will provide about 30 aliquots. Since about 84% of the test cells can be recovered following deglycerolization, and since these cells can be stored after deglycerolization at 4°C for about 2 weeks in .9% NaCl, a small amount of test cells will provide quality control material for about one year. This obviously is helpful in cost-containment approaches and guarantees availability of uniformly prepared test cells.

## CONCLUSIONS AND RECOMMENDATIONS

Although the contract year is not yet completed, much useful information has been gained. The principal conclusions and recommendations are as follows:

I) The new method to quantitate cell-bound C3 can be easily performed and confirms our previous findings that more C3b molecules are fixed to stored RBC than to fresh RBC. We also observed more C3d molecules on stored than on fresh RBC, although the stored RBC agglutinated less strongly with anti-C3d antibody than the fresh RBC. We will soon be able to determine exactly how many C3 molecules are present on stored RBC, fresh RBC, quality control RBC, platelets, or on RBC of patients who have various hemolytic anemias, etc. It is recommended that the new test be applied for purposes of performing these measurements.

II) The studies on the mechanism of complement activation under low ionic conditions have revealed the following information: when RBC and plasma are exposed to LISS at 37°C, complement system is activated by the classical pathway. The primary, initiating factor is binding of normal Ig to RBC membrane sites. These membrane sites can be deleted by prior treatment of RBC with bromelin. Most bound Ig elutes from RBC when ionic concentration is normalized, although some remains permanently RBC-bound. It appears that some Ig remain RBC-bound because they participate in the complement assembly to cell membrane, since in the absence of complement fixation (when heat-inactivated plasma was used) no significant amounts of Ig remained nonspecifically bound to RBC.

These data impact on three separate blood banking areas, and further study is recommended:

a) Basic theory of the low ionic strength method. Low ionic methods are used in blood banks for performing crossmatch; the methods have been developed on an empirical basis without a firm basic understanding. The approach described by us provides a model to study the sequence and interdependency of Ig and complement uptake by RBC in LISS. In particular, it is necessary to understand what conditions, if any, enhance the uptake of blood group antibodies without also causing uptake of Ig and complement, nonspecifically.

It is also necessary to know how many Ig molecules are retained by the disaggregated RBC nonspecifically and what is the subclass of the retained IgG. This will reveal whether their quantity is below the detection limit of the manual AGT, or whether the subclass and class specificity of these Ig permits their recognition by the antiglobulin sera.

This information would help utilize LISS in the optimal manner in the blood bank.

b) Purification and separation of antibodies using Rh-negative RBC. We have shown that blood group antibodies can be absorbed to and eluted from antigen-negative RBC. If antigen-positive RBC are used, antibodies can be absorbed to RBC but not eluted from them. Therefore, utilizing this approach and A and/or B RBC, which are negative for a

particular blood group antigen, isoagglutinins can be removed from various antisera. The antibodies can also be concentrated by varying the amount of the final eluate. It might be possible to exploit this methodology for preparation of Rh antibodies used to prevent Rh-hemolytic disease of the newborn. These methods could be also used for purposes of separating multiple antibodies from sera to facilitate antibody identification in the blood bank. The approach can also be used to demonstrate the presence of RBC antigens which are unable to support direct agglutination.

c) Studies on complement assembly. The model presented above permits a detailed study of the mechanism of complement assembly to RBC membrane. Only one study has recently indicated that Ig are involved in the initial complement assembly to cell membrane. Further studies in this area are recommended to confirm these findings.

III) Our studies have demonstrated that quality control cells can be preserved by freezing for prolonged periods. Utilizing this approach, it is possible to prepare a small quantity of carefully characterized test cells and thaw aliquots at two-week periods. Preparation of a 10ml aliquot is expected to last a year in an ordinary blood bank. It is recommended that blood banks adopt this procedure for their quality control procedures.

## REFERENCES

1. Szymanski IO and Odgren PR: Studies on the preservation of red blood cells. Attachment of the third component of human complement to erythrocytes during storage at 4°C. *Vox Sang* 36:213, 1979.
2. Graham MA, Davis DM, and Brower CE: Detection of C3b and C3d on red cell membranes; in *Nature and significance of complement activation*. pp.107-111 (Ortho Research Institute of Medical Science, Raritan, NJ, 1976).
3. Rosenfield RE, and Jagathambal: Antigenic determinants of C3 and C4 complement components on washed erythrocytes from normal persons. *Transfusion* 18:517, 1978.
4. Freedman J, and Massey A: Complement components detected on normal red blood cells taken into EDTA and CPD. *Vox Sang* 37:1, 1979.
5. Borsos T, and Leonard EF: Detection of bound C3 by a new immunochemical method. *Immunology* 107:766, 1971.
6. Fischer JT, Petz L, Garratty G, and Cooper NR: Correlations between quantitative assay of red cell-bound C3, serologic reactions, and hemolytic anemia. *Blood* 44:359, 1974.
7. Chaplin H Jr., Freedman J, Massey A, and Monroe ML: Characterization of red blood cells strongly coated in-vitro by C3 via the alternative pathway. In press, *Transfusion*.
8. Cines DB, and Schreiber AD: Immune thrombocytopenia. Use of a Coombs anti-globulin test to detect IgG and C3 on platelets. *New Engl. J. Med.* 300: 106, 1976.
9. Issitt PD, Issitt CH, and Wilkinson SL: Evaluation of commercial anti-globulin sera over a two-year period. I. Anti-beta 1A, anti-alpha 2D and anti-beta 1E levels. *Transfusion* 14:93, 1974.
10. Petz LD: Complement in Immunohematology and in neurologic disorders in: *The nature and significance of complement activation*. pp. 87-94 (Ortho Research Institute of Medical Science, Raritan, NJ, 1976).
11. Engelfriet CP: C4 and C3 on red cells coated in-vivo and in-vitro; in *The Nature and significance of complement activation*. pp.69-75 (Ortho Research Institute of Medical Sciences, Raritan, NJ, 1976).
12. Meryman HT, and Hornblower M: A method for freezing and washing red blood cells using a high glycerol concentration. *Transfusion* 12:145, 1972.
13. Marchalonis JJ: An enzymatic method for the trace iodination of immunoglobulins and proteins. *Biochem. J.* 113:299, 1969.
14. Gaither TA, Hammer CH and Frank MM: Studies of the molecular mechanisms of C3b inactivation and a simplified assay of B1H and the C3b inactivator (C3bINA). *J. Immunol.* 123:1195, 1979.

15. Mancini G, Carbonara AC, and Heremans JF: Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2:235, 1965.
16. Jenkins DE Jr., Moore WH, Luthringer DG, and Johnson RM: A rapid method for the production of erythrocytes in the state "EC4" and "EC43". *Transfusion* 15:402, 1975.
17. American Association of Blood Banks: *Technical Manual*, 7th ed. W.V. Miller, Ed., Washington DC, 1977.
18. Jenkins DE, and Moore WH: A rapid method for the preparation of high potency auto and alloantibody eluates. *Transfusion* 17:110, 1977.
19. Burkart P, Rosenfield RE, Hsu TCS, Wong KY, Nusbacher J., Shaikh SH, and Kochwa S: Instrumented PVP-augmented antiglobulin tests. I. Detection of allogeneic antibodies coating otherwise normal erythrocytes. *Vox Sang* 26:289, 1974.
20. Hsu TCS, Rosenfield RE, Burkart P, Wong KY and Kochwa S: Instrumented PVP-augmented antiglobulin tests. II. Evaluation of acquired hemolytic anemia. *Vox Sang* 26:305, 1974.
21. Szymanski IO and Huff S: unpublished data.
22. Stratton F, and Rawlinson VI: Preparation of test cells for the anti-globulin tests. *J. Clin. Path.* 27:359, 1974.
23. Garratty G, and Petz LD: The significance of red cell bound complement components in development of standards and quality assurance for the anti-complement components of antiglobulin sera. *Transfusion* 16:297, 1976.
24. Fruitstone MJ: C3b-sensitized erythrocytes. *Transfusion* 18:125, 1978.
25. Crosby WH, Munn JI, and Furth FW: Standardizing a method for clinical hemoglobinometry. *U.S. Armed Forces Med. J.* 5:693, 1954.
26. Jenkins DE, Jr., Hartmann RC, and Kerns AL: Serum-red cell interactions at low ionic strength: erythrocyte complement coating and hemolysis of paroxysmal nocturnal hemoglobinuria cells. *J. Clin. Invest.* 46:753, 1967.
27. Schumaker VN, Calcott MA, Spiegelberg HC, and Muller-Eberhard HJ: Ultracentrifuge studies of the binding of IgG of different subclasses to the C1q subunit of the first component of complement. *Biochemistry* 15:5175, 1976.
28. Muller-Eberhard HJ: The complement system in: *The plasma proteins. Structure, function and genetic control*. In 2nd ed. F.W. Putnam, Ed., New York, Academic Press, 1975, p.393.

29. Lachmann PJ, and Hobart MJ: Complement technology. In: *Handbook of experimental immunology*. 3rd ed., D.M. Weir, Ed., Oxford Blackwell Scientific Publ., 1978, p.5A.
30. Lachmann PJ: Complement. IN: *Clinical aspects of immunology*. 3rd ed., PCH Gill, RRA Coombs, and PJ Lachmann Eds., Oxford Blackwell Scientific Publ., 0.323, 1975.
31. Mollison PL, and Polley MJ: Uptake of gammaglobulin and complement by red cells exposed to serum at low ionic strength. *Nature (Lond.)* 203:535, 1964.
32. Goers JWF, and Porter RR: The assembly of early components of complement on antibody-antigen aggregates and on antibody-coated erythrocytes. *Biochem. J.* 175:674, 1978.
33. Rosenfield RE, Spitz C, Bar-Shany S, Permoad and, and Du Cros M: Low ionic concentration to augment hemagglutination for the detection and measurement of serological incompatibility In: *Automation in Analytical Chemistry (1967 Technicon Symposia)*, White Plains, Medical Inc., 1968, p.173.
34. Lalezari P: A new method for the detection of red blood cell antibodies. *Transfusion* 8:372, 1968.
35. Atchley WA, Bhagavan NV, and Masouredis SP: Influence of ionic strength on the reaction between anti-D and D-Positive red cells. *J. Immunol.* 93:701, 1974.
36. Moore HC, and Mollison PL: Use of a low-ionic strength medium in manual tests for antibody detection. *Transfusion* 16:291, 1976.
37. Abramson J, and Schur PH: The IgG subclass of red cell antibodies and relationship to monocyte binding. *Blood* 40:500, 1972.
38. Rosenfield RE, Shaikh SH, Innella F, Kaczera Z, and Kochwa S: Augmentation of hemagglutination by low ionic conditions. *Transfusion* 19:499, 1979.

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